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A Plasmid in *Streptococcus pneumoniae*

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Plasmid deoxyribonucleic acid has been detected in three related laboratory strains of *Streptococcus pneumoniae*. Strains D39S, R36, and R36NC each contain a minimum of two copies per cell of a 2.0-megadalton plasmid (pDP1). A plasmid twice as large as this smaller one is also present in much lower quantity in these strains, but neither plasmid is present in four strains related to these or in a drug-resistant clinical isolate from Paris. The plasmid yield was not amplified in the presence of chloramphenicol. No phenotype has been correlated with the presence of pDP1, which has existed in strains carried for many years in laboratory collections.

Although plasmids occur in several streptococcal species (12; V. Hershfield, Plasmid, in press), none had been observed in *Streptococcus pneumoniae* until recently. Dang-Van et al. (4) have suggested that the properties of drug-resistant strains isolated in Paris implied the presence of a plasmid, but they could not detect one by centrifugation methods. However, while examining multiply drug-resistant strains from South Africa (3, 8), L. Mayer (personal communication; L. W. Mayer and V. B. Ploscowe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, D26, p. 36) observed plasmid DNA in a culture of R36NC, a laboratory control strain he had received from R. Austrian.

We have extended the observation of Mayer by confirming the existence of the plasmid in R36NC, characterizing it, and tracing its ancestry to a clinical strain that was isolated by Avery at the Rockefeller Institute in 1916 (5) and is the progenitor of many widely used laboratory strains (1, 18), most of which no longer carry it as a detectable separate element.

MATERIALS AND METHODS

Bacterial strains. Pneumococcal strains D39S, R36, R36A, and R36NC were received lyophilized from R. Austrian. DP3105 was isolated from R36NC after growth in 0.5 μg of ethidium bromide (Calbiochem) per ml for four generations. Rx1 and R6 are derivatives of R36A which have been described previously (7, 18). BM6001 (4) was received from V. Hershfield.

Reference DNAs. DNAs from ColE1 (2) and PM2 (6) were gifts from P. Modrich and R. Porter, respectively. Lambda DNA was prepared by phenol extraction of CsCl-purified phage induced from strain B1042 (obtained from Modrich) and digested with *Eco*RI endonuclease, also obtained from Modrich.

Growth and lysis. Cells were grown to 10^9 cells per ml in a rich medium, CAT (16), plus for all strains except Rx1 an addition of 0.7% sterile filtered yeast extract (Difco Laboratories). They were diluted twofold into a solution containing 0.1 M Tris-hydrochloride, 0.01 M EDTA, 0.15 M NaCl, and 0.015 M sodium citrate (pH 7.5) at 0°C, centrifuged, and suspended at 10^{11} cells per ml in the same buffer plus 0.5% sodium deoxycholate. After 5 to 20 min at 37°C for cell lysis, sodium dodecyl sulfate was added to 1%, and the lysates were heated at 65°C for 15 min. R36NC and DP3105 were slow to lyse, and 0.1 volume of a fresh lysate of Rx1 was added to aid lysis (11).

Treatment of lysates. Crude lysates were brought to 1 M NaCl, let stand overnight at 0°C, and centrifuged for 20 min at 29,000 × *g*. The supernatant was gently extracted with redistilled phenol, added to 2 volumes of 100% ethanol at −20°C, let stand for 4 to 6 h at −20°C, and centrifuged. The precipitate was dissolved in electrophoresis buffer (see below) at 1/10 the volume of original lysate; DNase-free RNase A (Worthington Biochemicals Corp.) was added to a concentration of 10 μg/ml, and the mixture was incubated for 30 min at 37°C. After the addition of 10 μg of proteinase K (E. Merck) per ml, incubation was continued at 45°C for 30 min.

Dye-CsCl. A lysate of 5×10^{12} R36 cells treated as described above was centrifuged in CsCl-ethidium bromide gradients (17), and the bands containing covalently closed DNA were pooled and recentrifuged. The denser plasmid-containing band was collected and treated repeatedly with Dowex 50 to remove ethidium bromide (17).

Gel electrophoresis. Electrophoresis was carried out in agarose (Bio-Rad Laboratories) gels at 1.5 V/cm on a horizontal slab gel apparatus (13), using 89 mM Tris base-89 mM boric acid-2.5 mM disodium EDTA, pH 8.3, (TBE) as buffer. Before loading, samples (5 to 20 μl) were mixed with 20 μl of a solution containing 20% sucrose, 0.01% sodium dodecyl sulfate, 10% TBE, and 500 μg of bromocresol green (Sigma Chemical Co.) per ml. Gels were stained in 1 μg of ethidium bromide per ml in TBE for 1 h and photographed under shortwave illumination (UV Products model C61 source) through an orange filter (Hoya type 390) on Polaroid type 665 film.

RESULTS AND DISCUSSION

To confirm that a plasmid existed in strain R36NC, we independently obtained this and several other strains from R. Austrian and examined lysates of them by agarose gel electrophoresis as described above. Figure 1 shows that R36NC, R36, and D39S each contained plasmid DNA migrating faster than the smallest lambda DNA endonuclease *Eco*RI linear fragment. The plasmid was also detectable in lysates treated only with RNase and proteinase K (data not shown). Five strains did not contain plasmid DNA detectable by this analysis. These were R36A, R6, Rx1, BM6001, and DP3105, a strain isolated after growth of R36NC in ethidium bromide. Gentle shear of the lysates of these strains did not release a detectable DNA of unique size from the mass of chromosomal DNA (data not shown).

The genealogy of all of these strains except BM6001 is shown in Fig. 2. D39S, an encapsulated type II strain, was isolated from a patient by Avery in 1916 (5) and gave rise in the laboratory to the rough strain R36, from which R36A (1) and R36N (14) were isolated as colony morphology variants (R. Austrian, personal communication). R36NC is a competent derivative of R36N (14). R6 is a subculture of R36A selected in the 1950s for continued competence in the laboratory of R. D. Hotchkiss (personal communication), and Rx1 is descended from R36A by a very complex pathway (7, 18). It appears that in the transition from R36 to R36A the plasmid was either lost or, possibly, integrated into the chromosome. BM6001 is a type 19 drug-

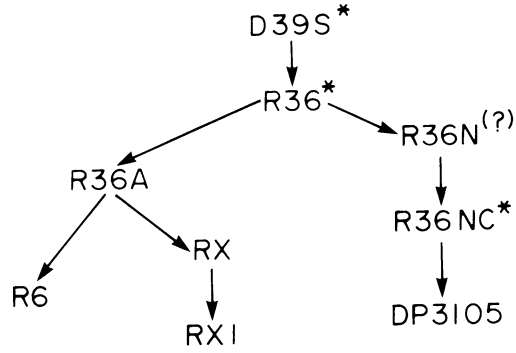

FIG. 2. *Genealogy and plasmid content of D39S and derivatives (see text). Asterisk indicates that the strain carries plasmids pDP1 and pX. R36N was not examined. Rx was derived from R36A through a complex series of steps described by Ravin (18). Rx1 designates a subculture carried for 20 years in this laboratory.*

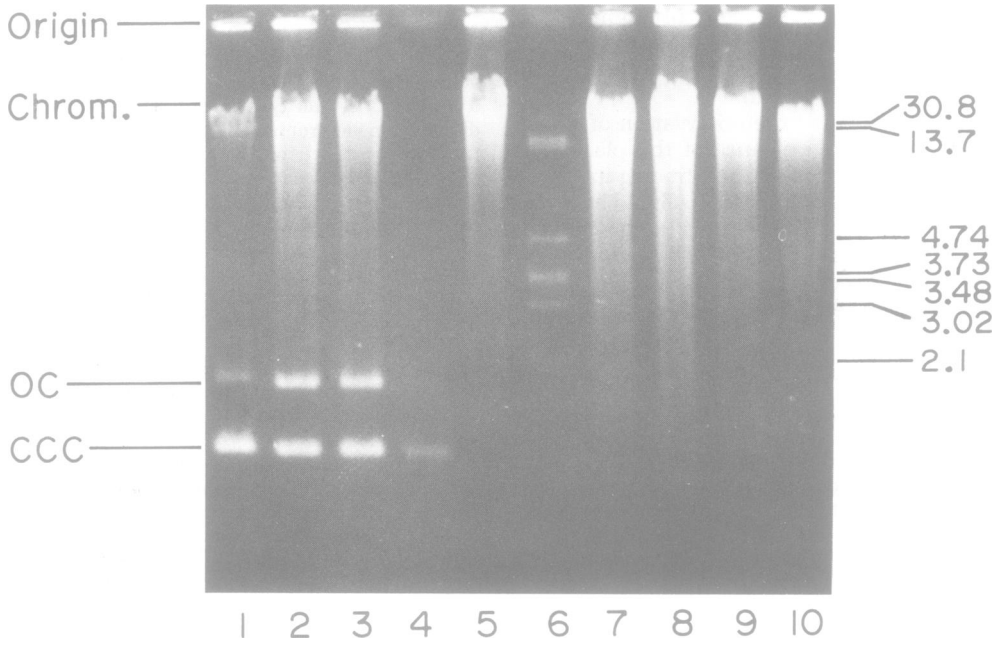

FIG. 1. *Agarose gel electrophoresis of treated lysates of pneumococcal strains. Except for lanes 4 and 6, each sample contains DNA extracted from 10^{10} cells (see text). Lane 1, D39S; lane 2, R36, lane 3, R36NC; lane 4, 12 ng of pDP1 DNA from R36; lane 5, Rx1; lane 6, 40 ng of lambda EcoRI digest; lane 7, R36A; lane 8, R6; lane 9, DP3105; lane 10, BM6001. Sizes of lambda EcoRI fragments (in megadaltons) are indicated (20). Chrom., Chromosomal DNA; OC, open circular DNA; CCC, covalently closed circular DNA.*

resistant clinical isolate from Paris described by Dang-Van et al. (4).

Lane 4 in Fig. 1 contains the plasmid purified from R36 by dye-buoyant density as described above. We define pDP1 as this plasmid in R36. The fastest moving species is the covalently closed form, as demonstrated by electron microscopy of the purified plasmid and by the fact that brief treatment with pancreatic DNase I converts the faster species to the slower one (data not shown). In lysates of all three strains that contain the small plasmid, a second pair of faint bands (temporarily designated pX) migrate more slowly, but slightly faster than ColE1 closed and open circles (data not shown), and DNase I also converts the faster form of pX to the slower one. After "curing" of R36NC to DP3105, both species disappeared. All species of plasmid appear to be identical in size among strains carrying them, and from their genealogy (Fig. 2) it is almost certain that they are the same in each strain.

Several further properties of pDP1 were examined as follows. (i) The contour length of open circle forms was compared with reference ColE1 and PM2 standards in electron micrographs, with the result (Table 1) that the size of pDP1 was determined to be 2.0 to 2.1 megadaltons. The migration of pX with ColE1 (4.2 megadaltons) on gels and its coexistence with pDP1 are therefore consistent with its being a dimer of pDP1. However, to prove this would require isolation of pX in quantities sufficient for restriction analysis. (ii) Two attempts to amplify the plasmid DNA in strain R36 by incubation of 5×10^8 cells per ml in a solution containing 10 μg of chloramphenicol per ml showed no increase of either plasmid or cell DNA in samples taken at 0, 2, 4, 8, and 16 h and analyzed on gels. (iii) The quantity of plasmid DNA recovered from a known number of cells was determined from the peak heights on densitometer tracings of the negatives of the gel photographs; these were standardized to *Eco*RI fragments of lambda DNA, and both closed and open circle forms were summed. Such determinations showed about two copies per cell in each strain, not significantly different from the number of chromosome copies per cell. Although inspection of gel photographs for crude lysates suggests that no large amount of plasmid was lost in the treatment of the lysate, this is of necessity a minimum estimate of the copy number. From renaturation kinetics the pneumococcal genome is 1.0×10^9 to 1.1×10^9 daltons (Smith, unpublished data), and therefore the recovered plasmid DNA was about 0.2% of the total DNA present in the cell. (iv) pDP1 is not cut by restriction endonuclease *Dpn*I, but is cut by *Dpn*II into at least three fragments, two of which are not well resolved on agarose gels (Fig. 3). Therefore, pDP1 is a unique species rather than a collection of similar-size molecules. Also, because R6, which does not have the plasmid, has DNA sensitive to *Dpn*II (10) and R36, which does have it, also makes DNA sensitive to this enzyme, the presence of the plasmid does not alter the complementary restriction nuclease specificity of these strains (10).

TABLE 1. *Size of pDP1*

Reference DNA	Size of reference DNA (megadaltons)	Mean length ratio of reference DNA to pDP1[a]	Size of pDP1 (megadaltons)
PM2	6.58 ± 0.18 (19)	3.14 ± 0.04	2.09
ColE1	4.2 (2)	2.09 ± 0.04	2.0

[a] Contour lengths of pDP1 and a reference molecule in the same electron micrograph field were measured five times each and averaged for each pair. The values shown are the mean and standard deviation of the ratios for six such pairs with each reference DNA. Grids were prepared essentially by the method of Kleinschmidt (9) and examined in a Japan Electronics and Optical Laboratories model JEM100C microscope.

Attempts to find a plasmid-correlated phenotype. No phenotype could be correlated with the presence of the plasmid pDP1. Phenotypes studied included bacteriocin production

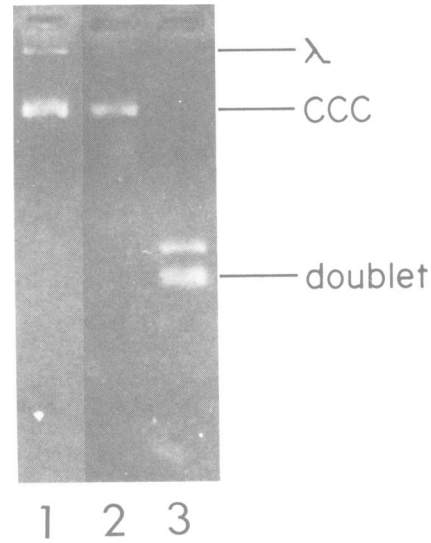

FIG. 3. *Action of nucleases DpnI and DpnII on pDP1. Gel electrophoresis was on 2.0% agarose for 8 h. The lanes all contained 20 ng of pDP1. Lane 1, untreated pDP1; lane 2, pDP1 treated with DpnI; lane 3, pDP1 treated with DpnII. Lane 1 also contained 10 ng of lambda DNA.*

(15), resistance to UV light (identical in R36 and R36A), phage susceptibility (R36, R36NC, R36A, and Rx1 were all susceptible to ω3 and ω8 [16]), and resistance to 13 metals and 38 antibiotics and other agents (Table 2). Although there were in some cases significant differences among the strains in susceptibility to these agents, in no case did they correlate with the presence or absence of the plasmid. Therefore, at this time pDP1 appears to be cryptic, although we have not yet ruled out the possibility that the plasmid DNA is integrated into the chromosome of those species not showing it as a separate circle.

The lack of resistance to antibiotics is not surprising in that the plasmid is found in a strain isolated long before the antibiotic era. Although no function has been detected, it has existed as a stable inherited part of the genome of pneumococcal strains that have been carried in laboratories for many years.

The recent appearance of multiply drug-resistant strains of pneumococcus (3, 4, 8) prompted a search for plasmids in this species. None was found in the South African strains (Mayer and Ploscowe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, D26, p. 36), and Dang-Van et al. failed to detect any in the Paris strain BM6001, although some properties of that strain suggested that a plasmid might be present (4). Our results (Fig. 1) confirm that no plasmid is detectable in BM6001 under conditions that readily detect pDP1 in the encapsulated strain D39S. Further studies on the resistance determinants of BM6001 and derivatives will be published elsewhere (Shoemaker, Smith, and Guild, manuscript in preparation).

TABLE 2. *Agents found not to discriminate between pneumococcal strains that contain circular pDP1 DNA and strains that do not*

Amethopterin	As_2O_3	Caffeine
Aminopterin	$AgNO_3$	Deoxycholate
Amphomycin	$BaCl_2$	Dinitrophenol
Ampicillin	$CaCl_2$	
Bacitracin	$Cd(NO_3)_2$	Hydroxyurea
Bryamycin	$CuSO_4$	KF
Chloramphenicol	$Fe_2(SO_4)_3$	Methylmethanesulfonate
Coumermycin	$HgCl_2$	Nitrosoguanidine
Erythromycin	LiCl	Proflavin
Fusidic acid	$MnSO_4$	Proteinase K
Hydroxyquinoline	$NiSO_4$	Sodium azide
Mitomycin C	VCl_3	Sodium dodecyl sulfate
Neomycin	$ZnSO_4$	Sodium hypochlorite
Nalidixic acid		Sodium nitrite
Novobiocin		6-(*p*-Hydroxy-phenylazo) uracil
Optochin		
Oxalinic acid		
Penicillin		
Quinacrine		
Rifampin		
Streptolidigin		
Streptomycin		
Sulfanilamide		
Tetracycline		

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